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Direct Synaptic Contacts of Medial Septal Efferents With Somatostatin Immunoreactive Neurons in the Rat Hippocampus

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YAMANO, M. AND P. G. M. LUITEN. *Direct synaptic contacts of medial septal efferents with somatostatin immunoreactive neurons in the rat hippocampus*. BRAIN RES BULL 22(6) 993–1001, 1989.—Anterogradely labeled projections from the medial septum to hippocampal somatostatin immunoreactive (SOM-i) neurons were studied with double-label immunocytochemistry under light (LM) and electron microscopic (EM) conditions. Medial septal projections were identified after injecting the anterograde tracer *Phaseolus vulgaris* leucoagglutinin (PHA-L) followed by immunohistochemical visualization of PHA-L presynaptic terminal labeling and concurrent immunocytochemical staining of SOM in hippocampal target cell bodies. This double-label procedure yielded blue-black nickel enhanced DAB stained, PHA-L-immunoreactive terminals on light brown SOM-i neurons that were investigated by correlative LM and EM observations. PHA-L-labeled terminal contacts with often basket-like appearance were localized with highest densities on soma and proximal dendrites of SOM-i neurons in stratum oriens of Ammon's horn and hilus of dentate gyrus, and some minor projections to stratum pyramidale and radiatum. Most double-labeled contacts could be identified as symmetric type synapses equally divided over soma and proximal dendrites of several forms of SOM-i neurons. These data indicate monosynaptic regulation of the hippocampal intrinsic SOM system by septal input, which probably represents a peptidergic subpopulation of the hippocampal GABAergic system.

Septo-hippocampal projections PHA-L tracing Double-label immunocytochemistry Somatostatin Ultrastructure

THE cornu ammonis (CA) and dentate gyrus (DG) of the hippocampal formation are recipients of a variety of extrinsic afferents from a number of sources that reach their target structures mainly via the perforant pathways and the fimbria-fornix systems (40,42). Several projecting systems have been described with increasing detail as a result of recent methodological improvements in retrograde and anterograde transport techniques for the detection of neuronal connectivity (5, 6, 16, 29, 34). Combining neuronal transport methods with immunocytochemistry revealed the transmitter type of several input systems and the position of a number of transmitter specific target structures (11, 22, 23, 32, 36). Within the hippocampus, neurons positively identified for containing gamma-aminobutyric acid (GABA), somatostatin (SOM), choline acetyltransferase (ChAT), vasoactive intestinal polypeptide (VIP), cholecystokinin (CCK) and a number of other peptidergic neuron types have now been reported (12, 32, 36, 37). Furthermore, there is well documented evidence for cholinergic and GABAergic input from the septum-diagonal band nuclei

widely projecting to all parts of the hippocampal formation, for hippocampal peptidergic fibers from the mammillary body and for various monoaminergic afferents from brainstem sources (1, 9, 13, 16, 18, 19, 21, 25, 26, 30, 39, 44, 45).

In a recent anterograde transport study employing the tracer *Phaseolus vulgaris* leucoagglutinin (PHA-L) (15) we obtained detailed anatomical information on the terminal and preterminal organization of septo-hippocampal projections (29). It was a striking observation that labeled terminal boutons were found embracing target neurons in the dentate hilus and stratum oriens of the cornu ammonis. This fiber morphology compared with the hippocampal distribution and shape of cells immunoreactive for SOM (32,36) prompted us to investigate PHA-L tracing of septo-hippocampal projections with concurrent immunocytochemical staining for presence of somatostatin. To gain more definite proof of tentative septal projections to SOM-immunoreactive target neurons in the hippocampus, we extended our analysis to the ultrastructural level to identify the synaptic nature of putative

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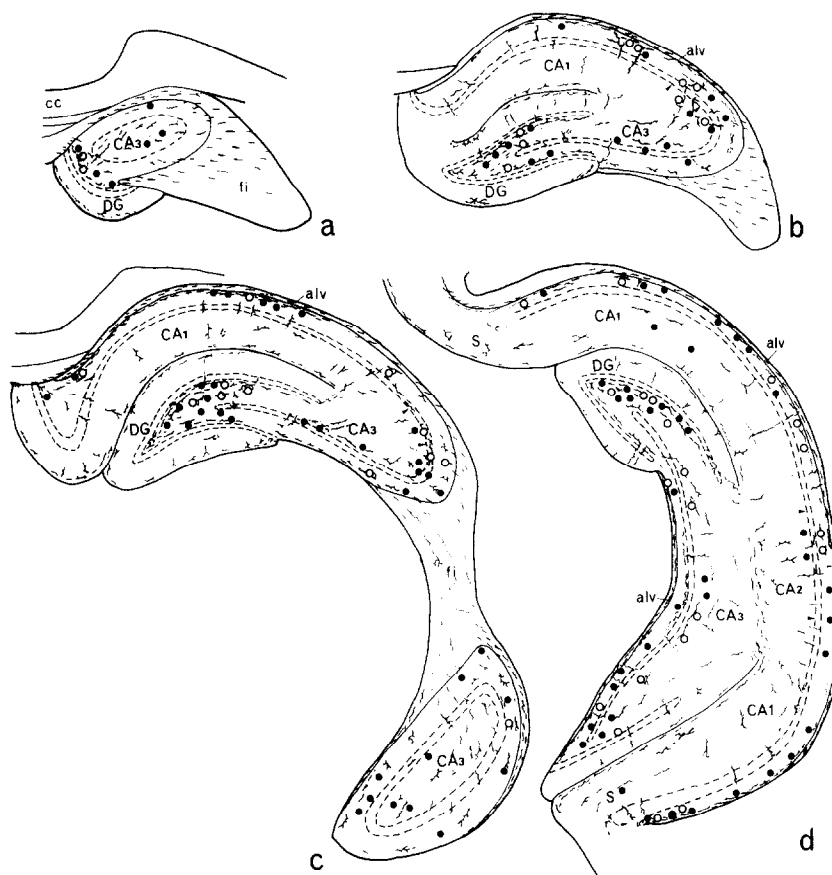


FIG. 1. Series of transverse hippocampal sections from anterior (a) to posterior (d) with the distribution of anterogradely labeled septo-hippocampal projections following a PHA-L injection in the medial septum. Cell bodies immunoreactive to somatostatin are indicated by open and filled circles and are mainly present in stratum oriens of the cornu ammonis and hilus of the dentate gyrus. Open circles in this experiment indicate SOM-immunostained cell bodies making direct synaptic contacts with PHA-L-labeled axon terminals. Abbreviations: CA1–3—cornu ammonis fields 1–3; alv—alveus; DG—dentate gyrus; S—subiculum; fi—fimbria; CC—corpus callosum.

intercellular contacts (23,46). Such a relationship of septo-hippocampal connections with SOMergic neurons may be particularly interesting because of the concurrent decline of the cholinergic component of the septo-hippocampal pathway and the SOMergic system in Alzheimer's disease. Furthermore, SOM immunoreactivity has been reported in neuritic plaques and neurofibrillary tangles which are the hallmarks of Alzheimer degenerations (2, 7, 8, 28, 33).

METHOD

The present investigation was based on six successful anterograde transport experiments with *Phaseolus vulgaris* leucoagglutinin (PHA-L) injected into the medial septum (MS), followed by double immunostaining for PHA-L-labeled fibers and cellular presence of somatostatin. All six animals were young male Wistar rats with a body weight of approximately 120 g. For PHA-L injection the animals were anesthetized with sodium pentobarbital (30 mg/kg IP) and positioned in a Narishige stereotaxic frame. The animals received a single PHA-L injection in the MS at coordinates AP: 9.5; L: 0 and V: 6.5 mm (31). The tracer was delivered iontophoretically via glass micropipettes with tip diameters between 15–20 μ m filled with 2.5% PHA-L (Vector labs) in tris

buffered saline. A positive-pulsed current 7 sec on in a 14 sec cycle for 30 minutes was provided by a Midgard CS-3 constant-current source. Six days after PHA-L injection, the animals received an intraventricular injection of 6 μ l of a colchicine solution (4 mg/ml) to enhance the concentration of SOM in the cell bodies. The next day the animals were perfused intracardially with 0.9% saline followed by 300 ml of fixative consisting of 4% paraformaldehyde, 0.08% glutaraldehyde and 0.2% picric acid in 0.1 M phosphate buffer (PB) at pH 7.4 (38). The brains were removed, postfixed overnight at 4°C in fresh fixative and subsequently cut to 40 μ m sections on a vibratome. For improved penetration of antibodies, the vibratome sections were immersed in 15% sucrose in 0.1 M PB followed by quick freezing in liquid nitrogen for 15 sec.

Double Immunostaining Procedure

The sections were first processed for immunostaining of PHA-L employing a goat anti-PHA-L antiserum (Vector Labs) (15,43). Prior to antibody treatment the sections were rinsed for 30 min in 10% normal donkey serum in PBS. Primary antibody incubation (dilution rate 1:2000) was carried out for 48 hr at 4°C. The second antibody donkey anti-goat IgG (1:200, Nordic Immu-

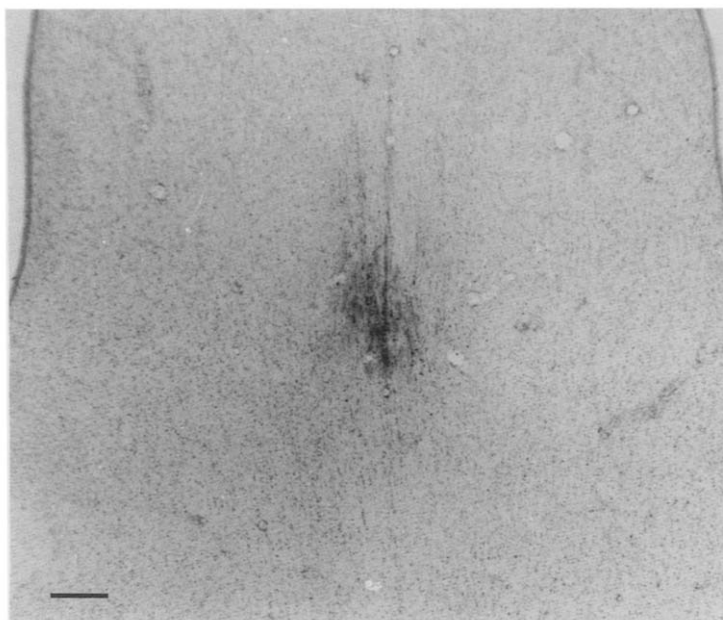


FIG. 2. Photomicrograph of a PHA-L deposit in the medial septum which can be identified by the PHA-L-labeled cell bodies in the midline region. Scale bar = 200 μ m.

nochemicals) was applied for 2 hr at RT, followed by exposure to goat peroxidase antiperoxidase complex (PAP, Dakopatts, 1:200) for 3 hr again at RT. The sections were then processed in 0.02% diaminobenzidine (DAB), 0.3% nickel ammonium sulphate and 0.005% hydrogen peroxide yielding a blue-black reaction product in the PHA-L-labeled terminals.

After immunostaining for PHA-L, the sections were incubated with 10% normal swine serum in PBS for 1 hr, followed by exposure to a rabbit anti-SOM antiserum (1:2000 in PBS, kindly donated by Dr. J. de Mey, Heidelberg) for 48 hr at 4°C. The primary antibody reaction was followed by subsequent incubations in swine anti-rabbit IgG (1:200, Nordic Immunochemicals) for 2 hr at RT and 3 hr in rabbit PAP complex (1:200, Dakopatts). The SOM immunoreaction products were visualized by a normal DAB reaction (0.02% DAB and 0.005% H_2O_2 in 0.05 M tris buffer, pH 7.6). This combined immunostaining procedure thus yields dark blue-black nickel enhanced DAB product in PHA-L-labeled axons including their terminal presynaptic boutons and light brown DAB precipitate in the SOM-positive cell bodies. Control experiments in which we omitted the primary antibody incubations or replaced it by normal sera resulted in total absence of any immune labeling.

The wet immunostained sections were observed under the light microscope and photographed. Then the sections were postfixated by 1% OsO_4 in 0.1 M PB, dehydrated in graded series of ethanol and flat-embedded in Epon 812. After light microscopic examination the tissue blocks were cut to ultrathin sections collected on single-slot grids coated with Formvar film and studied under a Philips 201 electron microscope.

RESULTS

Light Microscopic Observations

Injections of PHA-L in the medial septum resulted in the uptake of tracer and subsequent labeling of limited numbers of neuronal cell bodies which is indicative of the size of the injection (Fig. 2). From the injection site PHA-L-labeled axons can be followed to the hippocampal formation where a widespread but

also well-organized projection pattern can be distinguished similar to what we have previously reported (29). Labeled axons course via the fimbria fiber pathway, enter the alveus of the Ammon's horn from where the labeled axons penetrate into the various layers of CA1–3 and DG. Concurrent with previous findings it again became clear that the medial parts of the MS project to the dorsal (septal) pole of the hippocampus while lateral cell groups of the MS aim at more caudal (or temporal) parts of the hippocampal formation. In all cases, the strongest labeling of terminal axons occurred in the stratum oriens of CA1–3 and hilus of DG. Moderate labeling of presynaptic labeling was observed in the stratum radiatum, lacunosum-moleculare and dentate molecular layer and stratum pyramidale (Fig. 1). As usual with PHA-L tracting, labeled fibers could be followed over long distances up to the projection area where fiber branching occurred accompanied by local bud-like thickenings which are indicative for presynaptic boutons (19,37). In the presently followed PHA-L procedure, terminal and en passant boutons and varicosities were usually densely stained with the dark blue reaction product whereas the staining of fibers appears somewhat less intense. Furthermore, it was predominantly the thicker type fiber that became labeled (29). Probably this is the effect of lack of detergents in the incubation solutions which may hamper equally strong penetration of antibodies in terminals and axonal profiles.

The second immunostaining for SOM immunoreactivity (SOM-i) resulted in lightly brown staining in the same sections of many SOM-i cell bodies but not fibers due to the colchicine treatment. Similar to previous reports, the SOM-i neurons were found distributed, mainly in stratum oriens and hilus. Occasional SOM-i staining of cells occurred in the stratum radiatum and pyramidale mainly of CA2 and CA3 (Fig. 1). In many cases close basket-like contacts could be observed between the dark blue PHA-L-labeled endings and brown stained SOM-i cell bodies or their dendrites (Figs. 2–5). In the CA1 region SOM-i neurons with PHA-L-labeled contacts were mainly found in the dorsal parts of the hippocampus. The ventral CA1 also contains many SOM-i cells but with lower numbers of PHA-L-i contacts. This distribution is

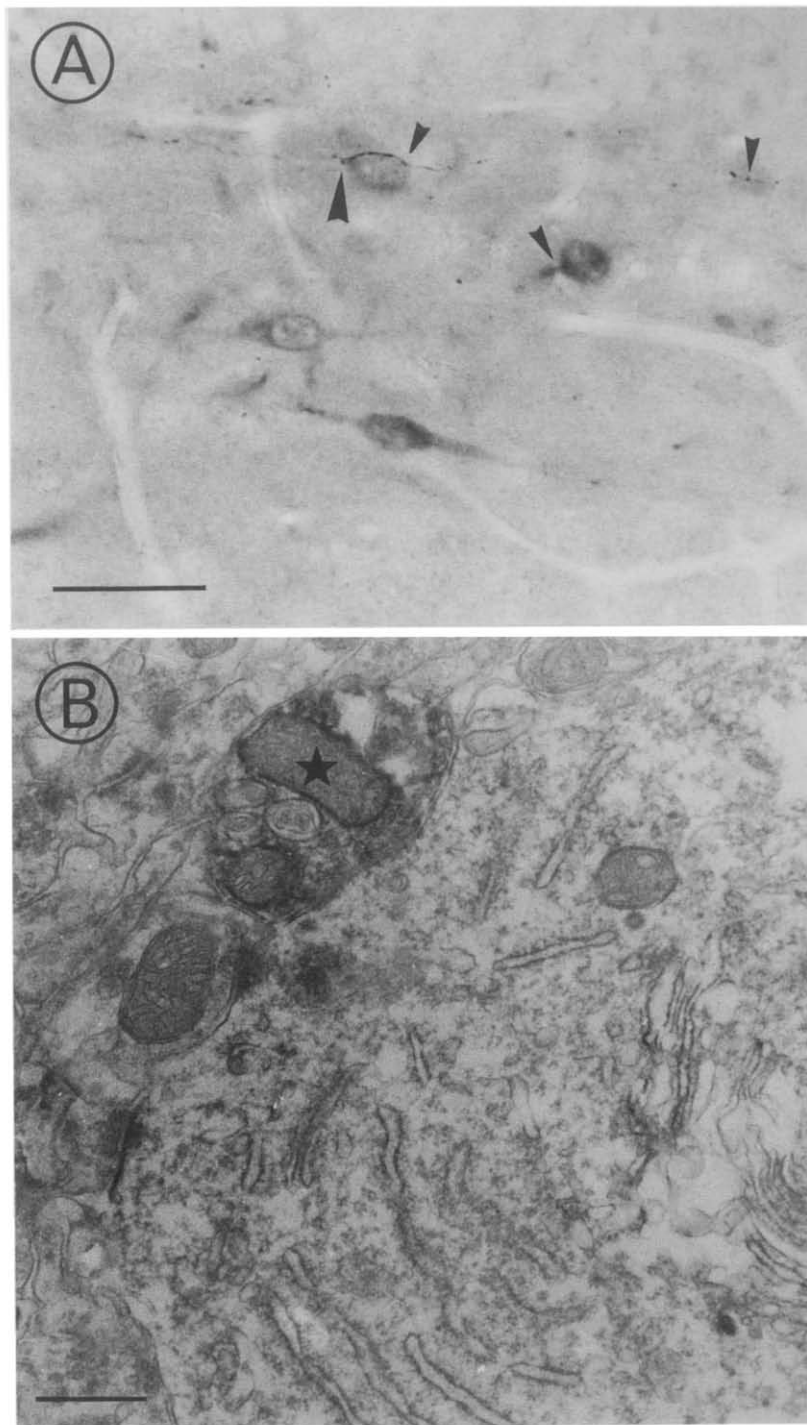


FIG. 3. (A) Light microscopic photograph of PHA-L-SOM double-label immunocytochemical demonstration of darkly stained PHA-L septo-hippocampal efferents and lightly staining SOM-positive cell bodies in the hilus of the dentate gyrus. At various sites intimate contacts between labeled efferents and SOMergic neurons (arrowheads) can be distinguished although not all in focus. Scale bar = 40 μ m. (B) Electron micrograph of PHA-L-immunoreactive presynaptic terminal (asterisk) and lightly SOM-immunoreactive soma of the axo-somatic contact shown in A by the large arrowhead. Scale bar = 1 μ m.

probably related to the more medial position of the PHA-L injection sites in the MS. Although some SOM-i cells in the CA1 area are also present in the stratum radiatum, contacts with

PHA-L-i terminals were only observed in the stratum oriens. In all cases PHA-L-i contacts consisting of multiple darkly stained varicosities were seen with round, multipolar or fusiform, hori-

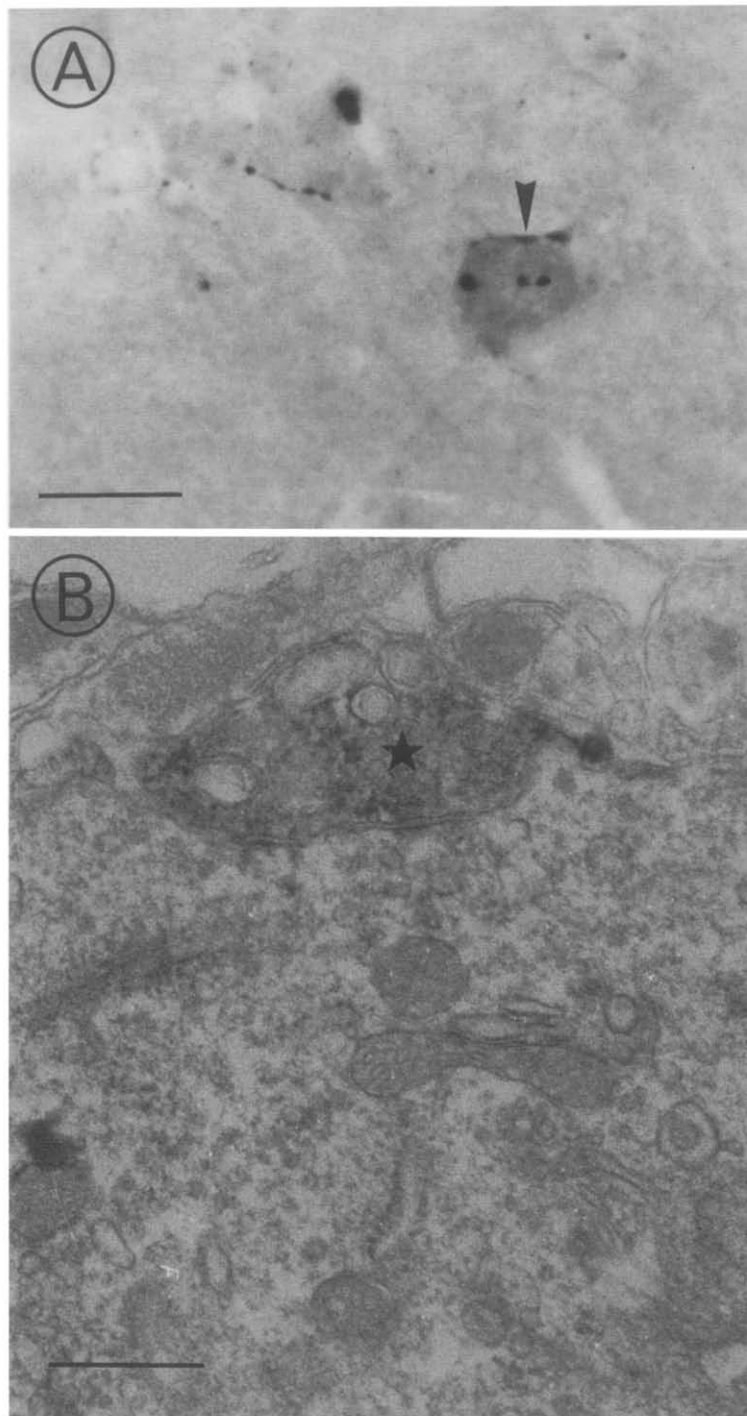


FIG. 4. Correlative LM (A) and EM (B) double-label immunocytochemistry of PHA-L-positive presynaptic endings on SOM-immunoreactive soma in the stratum oriens of CA1. Note a more darkly stained presynaptic terminal (arrowhead in A, asterisk in B) on a lightly immunoreactive soma making a symmetric type synaptic contact. Bar in A = 20 μ m, in B = 1 μ m.

zonally oriented SOM-i cells which did not appear different in type from the other noncontacted SOM-i cells. In the CA2-3 region, SOM-i neurons were widely present in the stratum oriens, but also in the stratum radiatum and pyramidale. Here, about half

of these SOM-i cells had PHA-L-i contacts which were somewhat less abundant in the ventral hippocampus. SOM-i neurons are numerous in the dentate hilus of which many were seen provided with PHA-L-labeled boutons and varicosities again more numer-

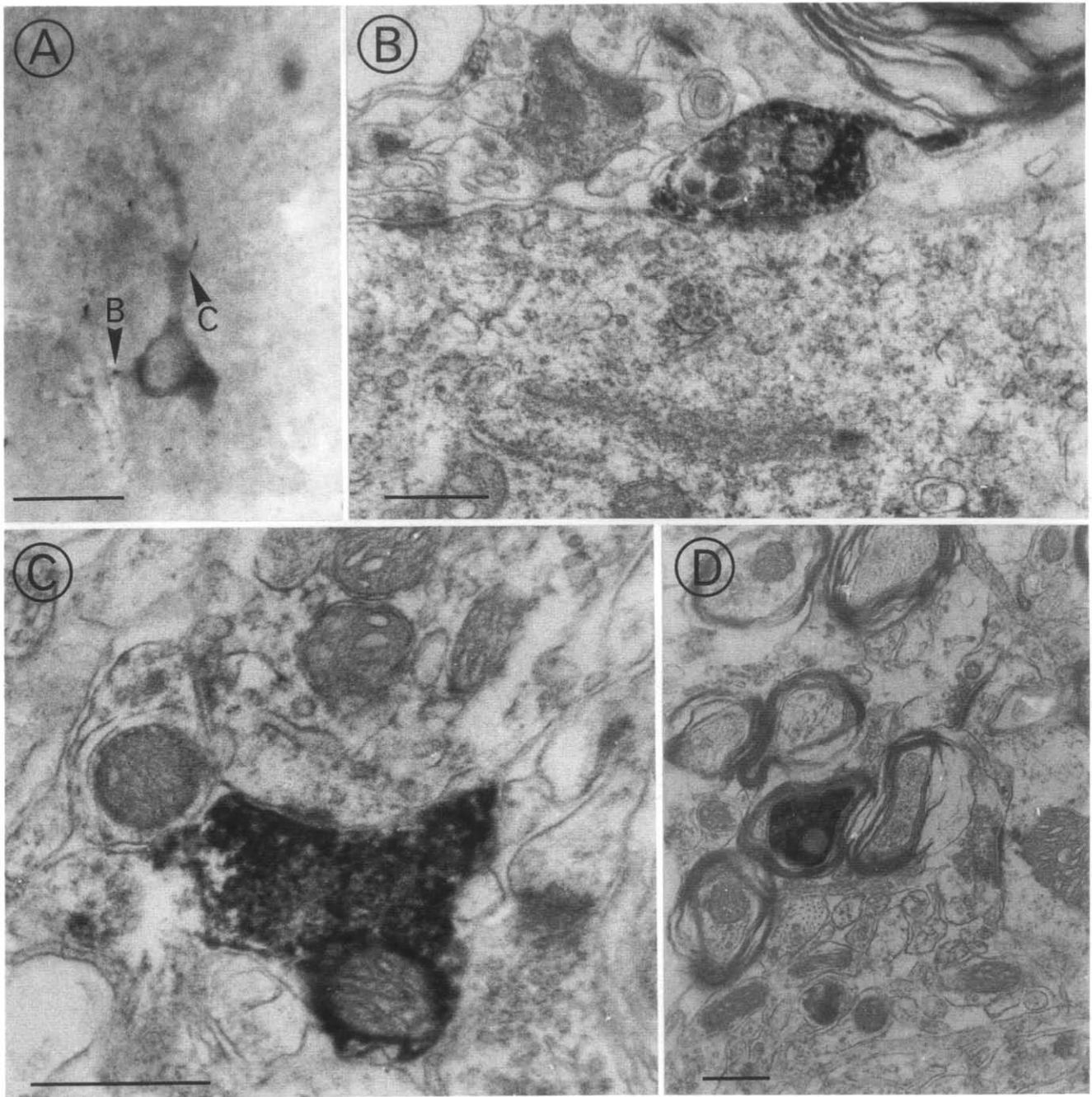


FIG. 5. LM and EM double-label immunocytochemical demonstration of PHA-L-labeled septo-hippocampal axon terminals contacting SOM-positive dendritic profiles of a multipolar interneuron in the stratum oriens of CA3. B and C are electron micrographs of the contacts indicated in A with arrowheads. D shows a PHA-L-labeled myelinated axon in the stratum oriens. Scale bar in A = 75 μ m, in B–D = 1 μ m.

ous in the dorsal aspects of the hippocampus.

Several of the observed double-immunolabeled contacts in hilus and Ammon's horn were selected for further electron microscopic analysis.

Electron Microscopic Observations

Characteristics of PHA-L-labeled fibers. As in the LM, PHA-L-labeled fibers could be followed over long distances in serial ultrathin sections. PHA-L-immunoreactive axons can easily be

detected under EM due to the relatively electrondense immunoreaction product in these fibers. In the fimbria and alveus most of the labeled fibers were myelinated, but in oriens, radiatum and hilus both myelinated and unmyelinated fibers occurred. The myelinated axons were thick, rich in axoplasm and well provided with mitochondria, whereas the unmyelinated fibers were very thin and delicate. The thick-labeled fibers in all cases were predominant over the thinner-labeled axons. Within the hippocampus the PHA-L fibers had swellings that resemble varicosities

observed at LM levels. These swellings under EM were filled with axoplasm, mitochondria and small vesicles, but it was not always clear whether these swellings are part of synaptic complexes. Near the SOM-i cells terminal PHA-L-labeled fibers sometimes were seen branching and forming swellings and boutons. These PHA-L-immunoreactive endings were provided with mitochondria and small vesicles, and very often observed to make synaptic contacts.

Synaptic Contacts of PHA-L-Labeled Terminals on SOM-i Neurons

The SOM immunoreactivity generally was rather light and could be recognized by randomly distributed DAB reaction products in the cytoplasm. Because of the difference in intensity in presynaptic PHA-L immunoprecipitate and the postsynaptic SOM-immune reaction, the LM and EM observation could easily be correlated. One example is illustrated in Fig. 4. Panel A shows the light micrograph of a SOM-i cell in the stratum oriens of CA3. In this case we observed six PHA-L-labeled boutons contacting the SOM-i cell body. Several ultrathin sections revealed all these contacts to form symmetric type synapses on the SOM-labeled cell body (Fig. 4B). This way 33 SOM-i cells in various parts of the hippocampus were investigated (Figs. 3–5). From these cases almost all PHA-L-labeled presynaptic contacts were localized on the soma or the main dendrite shafts (Fig. 5). We also consistently found that the great majority of SOM-positive cells received more than one PHA-L-labeled terminal even though the PHA-L injections in the medial septum were moderate in size. Moreover, although it was difficult to determine the classification of labeled synapses in asymmetric (Gray I) or symmetric (Gray II) type, most synapses appeared to be of the symmetric type at least as identified by the characteristic membrane specializations. It remains, however, difficult to identify the presynaptic membrane structures due to the often heavy presynaptic DAB precipitates. Another general observation was the almost equal proportion of axo-somatic and axo-dendritic contacts of labeled efferents to SOM-i cells. In many cases we identified PHA-L-labeled presynaptic contacts with nonlabeled postsynaptic profiles. In some cases there was some doubt on the SOM nature of the postsynaptic structure due to the light labeling especially in more distal aspects of the dendrites. Such cases, however, were rare. In all labeled SOM-i neurons, the cytoplasm was richly provided with endoplasmic reticulum, free ribosomes, Golgi complexes, mitochondria and an indented nucleus.

In the hilus of the dentate gyrus many SOM-positive nerve cells occurred and numerous PHA-L-positive fibers and boutons were seen at close proximity of these SOM cells. Frequently, clusters of boutons and varicosities were observed to embrace the SOM-i somata. SOM-immunoreactive neurons with those identified septo-hippocampal contacts were polymorphous and usually of a spherical or multipolar type but not of the basket cell type (Fig. 3). Many synapses were axo-somatic, but also axo-dendritic contacts were detected on the proximal main dendritic shafts near the cell soma (Fig. 3B). In most cases the synapses appeared of the symmetric type.

In the CA1 field most of the double-labeled synaptic contacts were observed in the stratum oriens especially close to the alveus from where the labeled axons reach the deeper hippocampal lamina. The often spherical and oval SOM-i neurons received many symmetric type PHA-L-labeled terminals mainly on the cell soma or on their proximal dendrites (Fig. 4). Multipolar SOM-i neurons also had many labeled symmetric type contacts on the large dendritic shafts near to the cell body.

The appearance of SOM-i neurons in the oriens of CA1 continues in the pre- and parasubiculum, but here the SOM-i neurons merge with the pyramidal cell layer. However, the SOM-i

cells are nonpyramidal and belong to the polymorphous or bipolar type. In the subicular areas, PHA-L-i contacts with SOM-i cells are of the same nature and morphology as in CA1.

In CA2 and CA3 many double-labeled synapses were seen in the stratum oriens, but here the labeled contacts appeared to be more widely distributed in other layers as well. SOM-i cells were detected in the pyramidal layer as well, also making contact with PHA-L-i terminals. These SOM-i neurons are not of the pyramidal type with its characteristic shape and orientation, but can be identified as having a multipolar form. In those cases most contacts were found on apical-like and basal dendrites making both symmetric and asymmetric type synapses (Fig. 5).

DISCUSSION

In the present investigation it was demonstrated that SOM-immunoreactive neurons in all parts of the hippocampus (including dentate gyrus and subiculum) are richly provided with direct synaptic input from the medial septal area via the septo-hippocampal pathway. In general the pattern of PHA-L-labeled septo-hippocampal connections fully agreed with the previously reported organization of this projection as established with retrograde and anterograde tracing methods (6, 25, 29, 35, 45). However, as compared to our previous report on PHA-L-labeled septo-hippocampal efferents (29), the present material appears to consist predominantly of labeled thick, so-called type I fibers. This more selective tracer labeling most likely is due to a reduced penetration of antibodies in the immuno-EM procedure used in the present study.

The transmitter nature of the anterogradely labeled projections visualized with PHA-L remains unknown. However, various studies employing combinations of tracing methods and transmitter immunocytochemistry indicate acetylcholine and GABA as the most likely candidates for these projections (1, 9, 21, 25, 26, 30, 45). In addition to such tracing studies, several lines of information indicate that both cholinergic and GABAergic septo-hippocampal systems project to SOMergic neurons. With regard to cholinergic projections it is clear that the laminar pattern of the PHA-L-labeled septal projection to stratum oriens, hilus, stratum peripyriformale and lacunosum-moleculare coincides well with the layered distribution of cholinergic marker enzymes (4, 18, 23, 24, 41). Moreover, previous ultrastructural investigation of choline acetyltransferase-labeled terminals revealed immunoreactive presynaptic contacts that for a part show to be identical to the PHA-L-labeled contacts found in the present study (10, 11, 22). On the other hand, it appears most likely that the contacts found labeled in the present study may only partly be cholinergic, and for the larger part GABAergic. A recent EM study employing combined staining of PHA-L-labeled septo-hippocampal efferents and GABA immunocytochemistry provide unequivocal evidence for GABAergic septal input to GABAergic target cells (9). Furthermore, these GABAergic septal projections, as in our material, were shown to form basket-like multiple contacts with the GABAergic target neurons. Likewise, their PHA-L-labeled efferents were predominantly of the thicker fiber type (9,23). Since SOM has been found colocalized with GABA for a very high percentage of SOM immunoreactive interneurons of the hippocampus, the septal projection may be interpreted as an input source to a SOMergic subpopulation of the intrinsic hippocampal GABAergic system (9, 36, 37). It is most likely that this GABA-SOMergic subpopulation, which is predominantly localized in the dentate hilus and hippocampal oriens, participates in the interhippocampal commissural system (22). It should be considered, however, that the double-labeling EM method used in this study will not visualize the entire tracer-labeled pathway. Previous ultrastructural studies revealed cholinergic terminals on

dendritic specialization which were not encountered in our material (10–12). The technical constraints of the double-label networks employed here may partly explain why synaptic contacts were not found on distal extensions of the dendritic trees. We should take into consideration that omission of detergents in the antibody incubation fluids will limit the penetration of antisera in the peripheral dendrites. Since our double-label material was selected on a positive identification in the LM of pre- and postsynaptic immunoprecipitates, certain distal dendrite contacts may have been neglected.

The now identified direct innervation of SOMergic hippocampal neurons by the septo-hippocampal system may be of great interest in the morphological substrate involved in the learning-memory function of the hippocampus. It has been well documented that experimental lesions of the septal-diagonal band region result in serious impairment of the animal's abilities in acquisition and retention of memory (17,27). In this respect such lesion studies have often been performed in relation to animal model studies for Alzheimer's disease (AD), which is characterized by cognitive impairment and deficiencies in cholinergic innervations of cortex and hippocampus originating from the

nucleus basalis and medial septal complex, respectively. As such, a direct connection between the medial septum and SOM-i neurons in the hippocampus may be of considerable significance because of the consistently found reduction of SOMergic systems in cortex and hippocampus in AD (8, 28, 33). To our knowledge, however, the relationship between a cholinergic or GABAergic septo-hippocampal input to SOMergic target neurons and memory function of the hippocampus is largely unclear. A first next step to undertake may be to investigate the effects of septal lesion on the structure of SOM and GABA neurons in the hippocampus as currently is investigated in our laboratory.

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